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"Indefinite for Dysplasia" in Barrett's Esophagus: Inflammation and DNA Content Abnormality are Significant Predictors of Early Detection of Neoplasia

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BACKGROUND: Dysplasia arising from Barrett's esophagus precedes esophageal adenocarcinoma (EAC). Cases that are difficult to diagnose as dysplastic, especially in the setting of inflammation, may be designated "indefinite for dysplasia (IND)." Although flow cytometric analysis of DNA content has shown some promise in detecting EAC, there are few reports that have specifically evaluated the outcome of IND.

AIMS AND METHODS: We analyzed a series of 96 IND patients seen at the University of Washington between 2005 and 2013 to determine the outcome of IND and to identify factors (including histologic features and DNA flow cytometric data) associated with subsequent detection of neoplasia.

RESULTS: Twenty-five percent of IND cases were found to have low-grade dysplasia, high-grade dysplasia (HGD), or EAC within 1 year, with 37% and 47% detected within 2 and 3 years, respectively. The 1-, 2-, and 3-year detection rates of HGD or EAC were 10%, 13%, and 20%, respectively. Active inflammation (hazard ratio (HR) = 3.4, $P = 0.0005$) and abnormal DNA content (HR = 5.7, $P = 0.003$) were significant risk factors of neoplasia. When active inflammation and DNA flow cytometric results were considered together, the HR for the combined markers was 18.8 ($P < 0.0001$). The sensitivity and specificity of the combined markers for predicting detection of subsequent neoplasia within 3 years were 100% and 60%, respectively, with 100% negative and 89% positive predictive values.

CONCLUSIONS: Histology with the support of DNA flow cytometry can identify a subset of IND patients who may have a higher risk for subsequent detection of neoplasia.

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INTRODUCTION

Barrett's esophagus (BE) is a major risk factor for the development of esophageal adenocarcinoma (EAC).[1–3] The incidence of EAC has continued to rise in the United States, at least until the year 2001. From 1975 to 2001, its incidence rose approximately sixfold, even outpacing those of melanoma, breast cancer, or prostate cancer.[4] Most EACs develop in the setting of BE through the chronic gastroesophageal reflux disease-metaplasia-dysplasia-carcinoma sequence.[1] The American College of Gastroenterology defines BE as endoscopically visible columnar epithelium extending upwards from the gastroesophageal junction that is histologically confirmed to have intestinal metaplasia (goblet cells), the conventionally accepted preneoplastic lesion for EAC in mucosal biopsy specimens.[3,5,6]

Multiple retrospective studies have demonstrated that EAC detected by endoscopic surveillance has lower staging with superior survival than EAC discovered without surveillance.[7,8] This observation has led to the current practice of endoscopic surveillance and therapy for BE patients, with the appropriate surveillance interval typically determined by the grade of dysplasia on an initial biopsy; more frequent surveillance and/or ablation therapy is needed with a higher grade of dysplasia.[3] Dysplasia in BE is defined as neoplastic epithelium confined to the basement membrane, and classified as negative for dysplasia, indefinite for dysplasia (IND), low-grade dysplasia (LGD), and high-grade dysplasia (HGD).[1] In LGD, there is a distinct lack of surface maturation with atypical nuclei limited to the basal portion of the cell cytoplasm while preserving crypt architecture.[1] In contrast, HGD is defined by the presence of full-thickness nuclear stratification, pleomorphism, atypical mitoses, and increased cytologic and/or architectural complexity, including back-to-back gland formations and increased crypt complexity.[1] The category of IND is used most often in the setting of inflammation/ulceration where a definite distinction between regeneration and dysplasia cannot be made with certainty. However, IND is also used when an accurate diagnosis cannot be made due to technical issues, including the lack of surface epithelium, marked cautery effect, or tangential section.[1] Interobserver variability in the diagnosis of dysplasia, especially IND, has been reported among pathologists.[9,10]

There are few reliable biomarkers/techniques to aid in differentiating non-dysplastic from dysplastic epithelium. Among the proposed markers for predicting malignant

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progression, the use of nuclear DNA content abnormities (such as aneuploidy) has shown some promise in predicting cancer risk.[1,3] Reid *et al.*[11] demonstrated that by using flow cytometry to measure the DNA content in the mucosa, patients who had negative, IND, or LGD biopsies without increased 4N or aneuploidy had a 0% 5-year cumulative cancer incidence, whereas patients with baseline increased 4N, aneuploidy, and HGD had 5-year cancer incidences of 56%, 43%, and 59%, respectively. However, in that study, DNA flow cytometry was evaluated as a potential objective marker for predicting progression to EAC, whereas in current clinical practice, determining risk for subsequent detection of LGD and HGD is equally important, as the grade of dysplasia dictates appropriate surveillance interval, and HGD or early EAC usually requires either nonsurgical ablation therapy or resection.[2,3,12]

Regarding IND, studies that have specifically evaluated the natural history or outcome of IND, including risk for subsequent detection of dysplasia or EAC, have been extremely limited. Available data on the neoplastic risk of IND have usually been provided only as a small subset of cases within the context of a larger dysplasia-endpoint study. In one study, Montgomery *et al.*[13] showed that ~12% of IND cases developed EAC within 3 years, similar to the 8% progression rate for LGD. However, these rates were based on only a single patient that progressed to neoplasia in each group.[13] Younes *et al.*[14] also reported that only 1 (2%) of 48 IND patients progressed to HGD or EAC after a mean follow-up of 41 months, but again, this was based on one patient that progressed to neoplasia, with limited follow-up time for the majority of 48 IND patients.

There are many endoscopic and pathologic factors that may affect the outcome results of IND, including the number of biopsies (sampling error), frequency/interval of surveillance endoscopy, and correct pathologic interpretation (dysplasia detected on the follow-up biopsies of IND being true dysplasia in the first place vs subsequently developed, histologically confirmed dysplasia).[1] As a result, the management of IND with endoscopic surveillance varies among different institutions. In fact, the guidelines for the diagnosis, surveillance, and therapy of BE issued by the American College of Gastroenterology (in 2008)[3] and the American Gastroenterological Association (in 2011)[2] do not provide specific guidance for the management of IND.

This study sought to determine the outcome of IND and to identify factors predictive of subsequent detection of dysplasia or EAC occurring within 3 years of initial IND biopsy. We analyzed follow-up data on 96 established BE patients who had diagnoses of IND between 2005 and 2013. Specifically, we aimed to correlate subsequent detection of LGD, HGD, or EAC with histological active (neutrophilic) inflammation, DNA flow cytometric data (aneuploidy and/or increased 4N fraction), and endoscopic findings in a statistically rigorous manner.

METHODS

Patients and data collection. Using our pathology information system (Power Path, Sunguest, Tucson, AZ, USA), we performed a review involving 96 patients with histologically confirmed Barrett's metaplasia who were categorized as having IND and had follow-up biopsies, collected at the University of Washington and Harborview Medical Centers between 2005 and 2013. Our study was approved by the University of Washington Institutional Review Board for human subjects research, #46704. Table 1 shows demographic characteristics of this cohort. For inclusion in this study, patients with a diagnosis of IND must have had follow-up biopsies. One endpoint of this was subsequent histological detection of any neoplasia, including LGD, HGD, or EAC; the second endpoint was subsequent histological detection of higher-grade neoplasia, comprising HGD or EAC. Hospital clinical and Power Path electronic medical records were further reviewed for these patients, and we retrieved pertinent data, including active (neutrophilic) inflammation in the area of IND, DNA flow cytometric data (aneuploidy and/or increased 4N fraction), and endoscopic findings (length of BE segment (short <3 cm and long >4 cm of Barrett's mucosa), nodule/nodularity, and hiatal hernia). All 96 IND cases were reviewed by gastrointestinal pathologists at the University of Washington Medical Center. IND was defined as an area of

Table 1 Characteristics of 96 patients diagnosed with IND at the University of Washington and Harborview Medical Centers between 2005 and 2013

	Entire cohort ($N=96$)	Cohort with baseline inflammation ($N=46$)	Cohort without baseline inflammation ($N=50$)
Mean age, years (range)	63 (39–86)	62 (39–86)	63 (43–82)
Gender	75 males, 21 females	36 males, 10 females	39 males, 11 females
Mean weight, kg (range)	87 (54–134)	88 (58–131)	87 (54–134)
Mean BMI, kg/m^2 (range)	28 (19–48)	29 (19–48)	27 (21–34)
Race	55 Caucasians 41 non-Caucasians	21 Caucasians 25 non-Caucasians	34 Caucasians 16 non-Caucasians
PPI use	64% (61/96)	54% (25/46)	72% (36/50)
Median time interval, months to first surveillance following IND (range)	7 (0.5–49)	5.5 (0.5–39.5)	9 (0.5–49)
Median time interval between all endoscopies, months (range)	8.5 (0.5–60)	7 (0.5–40.5)	12 (0.5–60)
Mean number of follow-up endoscopies from IND to LGD/HGD/EAC (range)	1.7 (1–6)	1.9 (1–6)	1.4 (1–4)

BMI, body mass index; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; IND, indefinite for dysplasia; LGD, low-grade dysplasia; PPI, proton pump inhibitor.

atypical surface and crypt epithelium suggestive of possible dysplasia, but with interpretation limited due to associated inflammation (raising the possibility of reactive atypia), ulceration, or technical issues, including lack of surface epithelium, marked cautery effect, or tangential sectioning, reducing the certainty of the evaluation of the biopsy.[1]

DNA content flow cytometry. Among the 96 IND cases, 39 patients (41%) had concurrent flow cytometric DNA analysis. There is no established guideline on the appropriate use of DNA flow cytometry for the management of BE patients. Therefore, DNA flow cytometric analysis was performed for a variety of reasons, including the provider's clinical suspicion of dysplasia based on endoscopic findings, easy access to the DNA flow cytometry laboratory at the University of Washington, potential applications of DNA content abnormalities in predicting cancer risk, and/or the patient's desire for the testing. As described previously,[11] one half of the biopsy specimen was fixed in formalin for histological examination, and the other half was processed for flow cytometry and analyzed by the computer program Multicycle (Phoenix Flow Systems, San Diego, CA). An aneuploidy population was defined and described previously.[15,16] The finding of 4N fractions greater than 6% of the nuclei (within the range of 3.85N to 4.1N) was classified as abnormal. Flow cytometric histograms were interpreted by one pathologist (PSR) blinded to the histologic results.

Statistical analysis. Statistical analysis was performed using methods appropriate for censored data (Kaplan–Meier curves (KM) and the Cox proportional hazards model), as follow-up time varied among patients (minimum = 0.3 months and maximum = 88 months). The presence of active inflammation and abnormal DNA flow cytometric findings were assessed using both univariate and multivariate Cox models. The KM and Cox analysis utilized the entire follow-up time period available for each person; the KM provides estimates of detection over the entire range of follow-up times with larger confidence intervals (CIs) as fewer people become at risk at later times, and the hazard ratio (HR) estimate in the Cox proportional hazards model is a weighted average of the hazard at each failure time, weighted according to the number of individuals still at risk.[17] Analysis was performed using the "survival," "rms," and "Hmisc" packages in R (www.r-project.org). For primary analyses, only patients with the complete data were included and reported. Because a significant number of patients did not have DNA flow cytometry assessed at baseline IND diagnosis, we used a multiple imputation method to obtain an unbiased estimate of the HR by jointly estimating the missing data for 23 individuals who had no DNA flow cytometric analysis and were negative for active inflammation at baseline. Briefly, the probability of a DNA flow cytometric abnormality for each of these 23 patients was calculated using Bayes' Theorem on the basis of his/her censoring or event time and the KM estimates of detection from the complete data only.[18] Missing data were drawn from these distributions for imputations ($N = 1000$), and the HR was calculated for each draw, providing a mean HR as the unbiased imputation estimate. The variance estimate for the imputation-estimated HR was calculated as a sum that incorporated the error variability from the observed data plus the error variability introduced by estimating the missing DNA flow cytometric measurements.[18]

The results from the imputation analysis were compared with the KM estimates obtained from the conservative method of classifying all patients with missing DNA flow cytometric data as negative for DNA content abnormalities. This constitutes misclassification of patients as normal who might not be normal, which is well known to result in attenuated/conservative estimates of risk (HR estimate that is biased downward from the truth, less separation between groups and a larger *P*-value).[19] This latter method allows one to assess the effects of the multiple imputation analysis relative to the conservative assumption of no DNA flow cytometric abnormalities among the 23 patients. Furthermore, the misclassification method parallels the reality of the current practice in BE and provides an estimate of the actual predictive power of the DNA flow cytometry combined with active inflammation over a number of clinics that do not have access to a DNA flow cytometry laboratory, while information on active inflammation is available. The CIs for the imputation-based estimate are no more narrow than for the estimate from the analysis that (incorrectly) assumes all missing DNA flow cytometric measurements were negative, as no more real data are obtained via imputation, but the imputation-based estimate is shifted away from the biased estimate obtained under this incorrect assumption.

For estimates of 1-, 2-, and 3-year detection/progression and for the overall detection/progression KM estimates, only patients having at least one follow-up endoscopy within the respective time interval or who were known not to progress in that interval were included in the risk set. There were $N = 70$, $N = 48$, and $N = 42$ patients with appropriate follow-up time for estimates of 1-, 2-, and 3-year detection/progression estimates for dysplasia or EAC. The 42 patients who had at least 3 years of follow-up were used to estimate 3-year sensitivity, specificity, positive predictive value, and negative predictive value of the combined active inflammation and DNA flow cytometric markers for subsequent detection of LGD, HGD, or EAC. The CIs for these proportions were obtained by Wilson's method.

RESULTS

Detection of LGD, HGD, or EAC. The overall demographic characteristics of the cohort and those divided by the presence or absence of active inflammation at baseline are shown in Table 1. The use of proton pump inhibitor was slightly higher among patients without active inflammation at baseline, but the difference was not statistically significant between cohort with and without baseline active inflammation ($P = 0.09$). Mean and median follow-up times were 14 and 10 months, respectively, for the 96 IND patients (minimum = 0.3 months and maximum = 88 months). Regarding follow-up, 70, 48, and 42 patients had follow-up time appropriate for 1-, 2-, and 3-year risk estimates, respectively, for subsequent detection of dysplasia or EAC. A total of 35 patients had histological evidence of LGD, HGD, or EAC. The

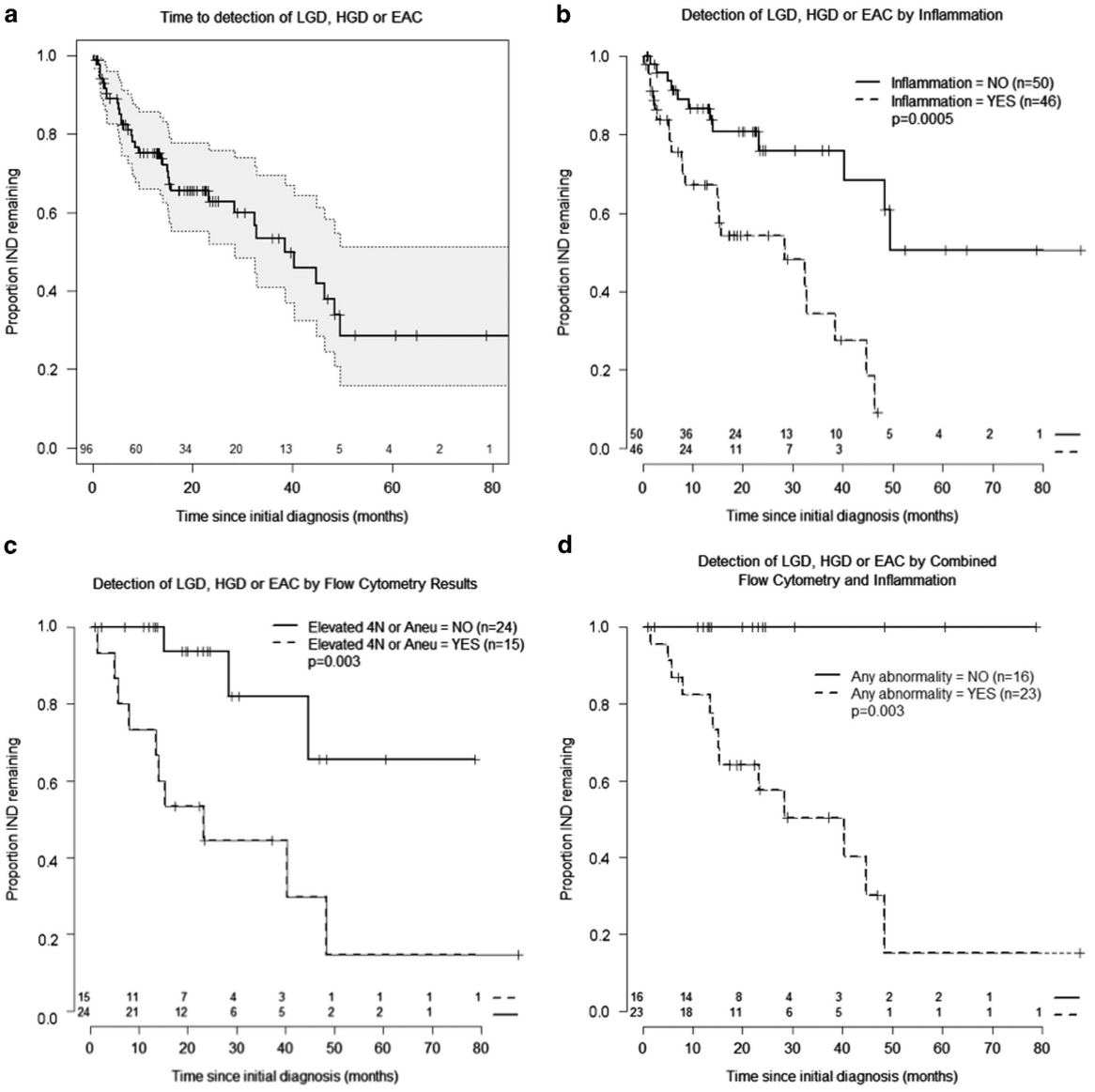

Figure 1 (**a**) Overall detection of LGD, HGD, or EAC in BE patients with IND; (**b**) detection of LGD, HGD, or EAC in IND patients with active inflammation at baseline; (**c**) detection of LGD, HGD, or EAC in IND patients with abnormal DNA flow cytometric results at baseline (either elevated 4N fraction or aneuploidy); and (**d**) detection of LGD, HGD, or EAC in IND patients with either a DNA flow cytometric abnormality or active inflammation at baseline. Each tick represents a person being censored, and the number of people remaining at risk at each 10-month interval is indicated below that axis. The estimates after 60 months have wide CIs (thus more uncertainty). BE, Barrett's esophagus; CI, confidence interval; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; IND, indefinite for dysplasia; LGD, low-grade dysplasia.

KM analysis showed that 1-, 2-, and 3-year detection rates of LGD, HGD, or EAC were 25%, 37%, and 47%, respectively (95% CIs = (14%, 34%), (24%, 48%), and (31%, 60%), respectively, Figure 1a). The presence of active inflammation at baseline (in 46 of 96 patients) was found to be a highly significant predictor of early detection of LGD, HGD, or EAC (Figure 1b; Table 2). The univariate HR associated with active inflammation was estimated to be 3.4 from the Cox model ($P = 0.0005$, 95% CI = (1.7, 7.5); Table 2).

Both aneuploidy and elevated 4N fraction were also associated with an increased risk of subsequent detection of dysplasia or EAC. Patients with aneuploidy had an estimated HR of 4.0 ($P = 0.007$, 95% CI = (1.4, 12.1)), whereas patients with elevated 4N fraction had an HR of 4.4 ($P = 0.005$, 95% CI = (1.4, 13.6); Figure 1c,Table 2). For patients who had either DNA flow cytometric abnormality or active inflammation, the difference in the detection rate of neoplasia was highly significant ($P = 0.003$) among the 39 patients who had DNA flow cytometric data available, but the HR was not estimable (infinite), because no patients without any of the abnormalities were found to have dysplasia or EAC during follow-up (Figure 1d,Table 2). Patients with neither active inflammation nor a DNA flow cytometric abnormality had a stable 1-, 2-, and 3-year detection rates of 0%, whereas patients with any of these risk factors had 1-, 2-, and 3-year detection rates of 18%, 45%, and 50%, respectively (Figure 1d).

Table 2 Univariate and multivariate Cox proportional hazards models with LGD/HGD/EAC or HGD/EAC as the outcome

Univariate model variable	Group (*N*)	Outcome					
		Detection of LGD/HGD/EAC			Detection of HGD/EAC		
		P value	HR	95% CI	*P* value	HR	95% CI
Inflammation No	50						
Inflammation Yes	46	0.0005	3.4	(1.7, 7.5)	0.003	5.8	(1.6, 21.4)]
Aneuploidy No	31						
Aneuploidy Yes	8	0.007	4.0	(1.4, 12.1)	0.13	3.8	(0.6, 23.7)
Elevated 4N No	27						
Elevated 4N Yes	12	0.005	4.4	(1.4, 13.6)	0.84	1.2	(0.2, 7.3)
Elevated 4N or aneuploidy No	24						
Elevated 4N or aneuploidy Yes	15	0.003	5.7	(1.6, 20.9)	0.13	4.8	(0.5, 43.5)
Age >60 No	44						
Age >60 Yes	52	0.58	0.8	(0.4, 1.6)	0.28	0.57	(0.21, 1.6)
Inflammation or flow abnormality No	16						
Inflammation or flow abnormality Yes	23	0.003	Infinite	NA	0.11	Infinite	NA
Inflammation or flow abnormality No*	39						
Inflammation or flow abnormality Yes*	61	<0.0001	18.8	(4.5, 79)	<0.02	10.3	(1.7, 63)
BE length long No	29						
BE length long Yes	30	0.43	1.4	(0.6, 3.4)	0.19	2.5	(0.6, 10.4)
Nodule No	52						
Nodule Yes	7	0.97	1	(0.2, 4.2)	0.93	1.1	(0.1, 8.9)
Hernia No	22						
Hernia Yes	37	0.55	1.4	(0.5, 3.7)	0.66	1.4	(0.3, 6.9)
Multivariate Cox model		**(*N* = 39, 12 events)**			**(*N* = 39, 5 events)**		
Inflammation		0.02	4.9	(1.4, 17.8)	0.04	15.1	(1.1, 213)
Any flow abnormality		0.002	9.7	(2.3, 40.1)	0.06	11.5	(0.8, 157)

BE, Barrett's esophagus; CI, confidence interval; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; HR, hazards ratio; LGD, low-grade dysplasia; NA, not applicable
*Based on a multiple imputation (unbiased) menthod.

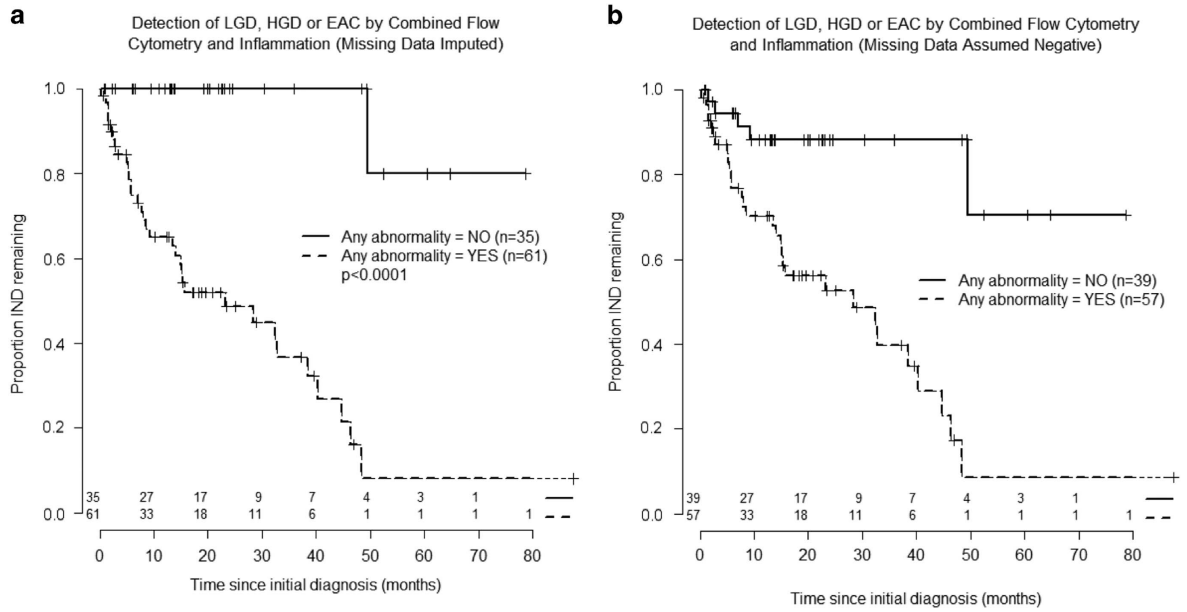

Figure 2 (**a**) Detection of LGD, HGD, or EAC in IND patients with either a DNA flow cytometric abnormality or active inflammation at baseline, using a multiple imputation (unbiased) method; and (**b**) detection of LGD, HGD, or EAC in IND patients with either a DNA flow cytometric abnormality or active inflammation at baseline, assuming that patients without DNA flow cytometric data had no DNA content abnormalities at baseline. This assumption increases the sample size relative to the Figure 1d (thus, providing more statistical power to reject the null hypothesis for the combined markers), but diminishes the true difference between groups due to measurement error. Each tick represents a person being censored, and the number of people remaining at risk at each 10-month interval is indicated below that axis. The estimates after 60 months have wide CIs (thus more uncertainty). CI, confidence interval; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; IND, indefinite for dysplasia; LGD, low-grade dysplasia.

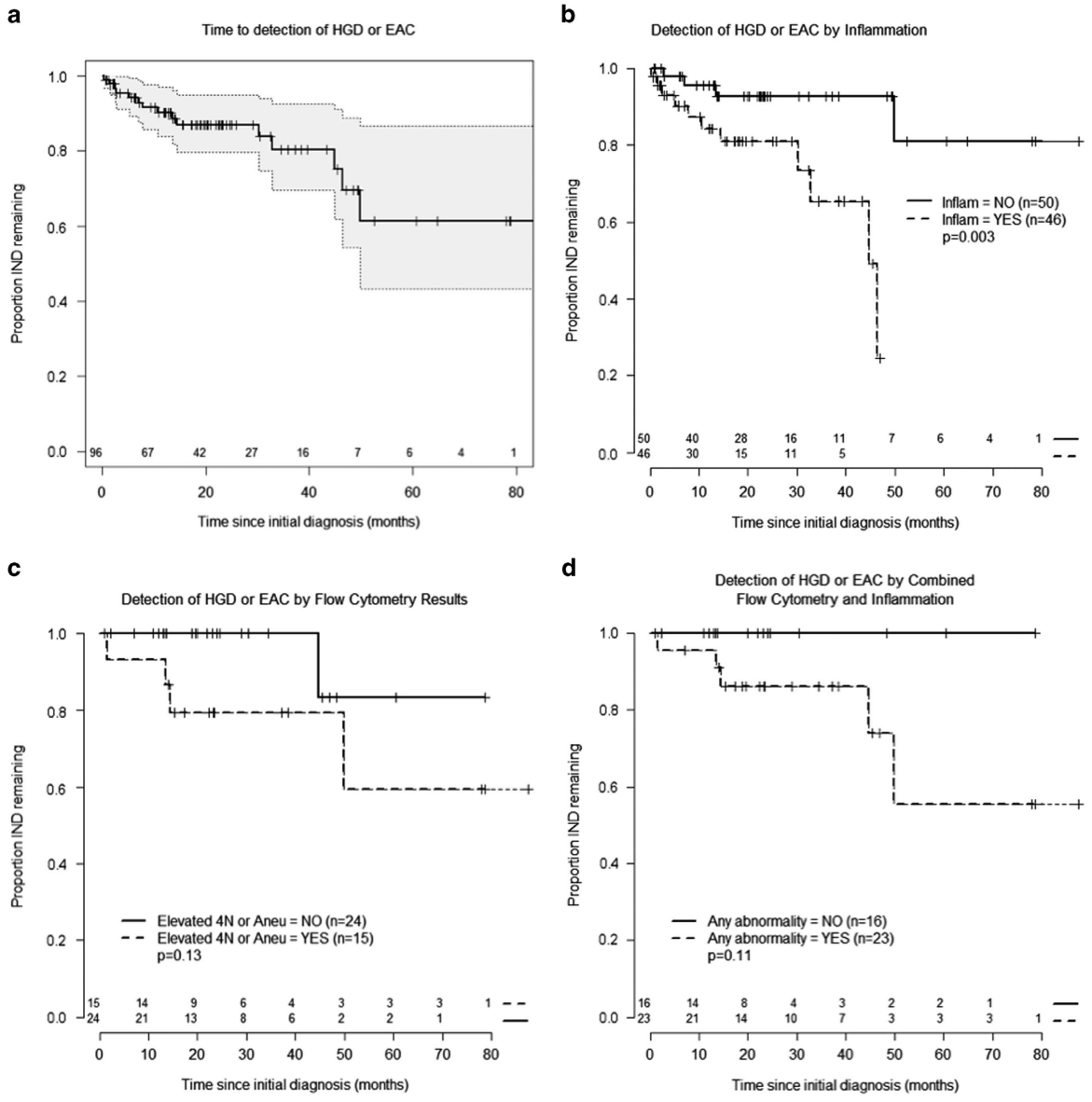

Figure 3 (**a**) Overall detection of HGD or EAC in BE patients with IND; (**b**) detection of HGD or EAC in IND patients with active inflammation at baseline; (**c**) detection of HGD or EAC in IND patients with abnormal DNA flow cytometric results at baseline (either elevated 4N fraction or aneuploidy); and (**d**) detection of HGD or EAC in IND patients with either a DNA flow cytometric abnormality or active inflammation at baseline. Each tick represents a person being censored, and the number of people remaining at risk at each 10-month interval is indicated below that axis. The estimates after 60 months have wide CIs (thus more uncertainty). BE, Barrett's esophagus; CI, confidence interval; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; IND, indefinite for dysplasia.

To obtain an estimate of the HR for the combination markers of active inflammation and DNA flow cytometric abnormality, a multiple imputation (unbiased) method was used with a large number of imputations (1,000) to account for imputation error, resulting in an estimated HR of 18.8 ($P<0.0001$, 95% CI = (4.5, 79); Figure 2a,Table 2). For comparison, all patients with missing DNA flow cytometric data were conservatively classified as negative for DNA flow abnormalities, and the KM plots were redrawn. This conservative classification method results in estimates of the HR and separation between groups that are biased downward relative to knowing the true DNA flow cytometric measurements. The comparison shows a slight but meaningful difference (Figure 2b): 39 of 96 patients were classified as having neither active inflammation nor DNA flow cytometric abnormality with the conservative classification (HR = 5.7 for the latter), whereas an average of 35 patients were classified as having no abnormality in the imputation analysis. As expected, the imputation method estimates that 4 additional patients had DNA flow cytometric abnormalities, on average, among the 23 missing DNA flow cytometric data with no active inflammation at baseline. Using only patients whose first follow-up status was known within 3 years ($N=42$), estimated sensitivity of the combined markers for detection of LGD, HGD, or EAC within 3 years was 100% (95% CI = (91%, 100%)) with a specificity of 60% (95% CI = (31%, 83%)), 100% negative predictive value (95% CI = (61%, 100%)), and 89%

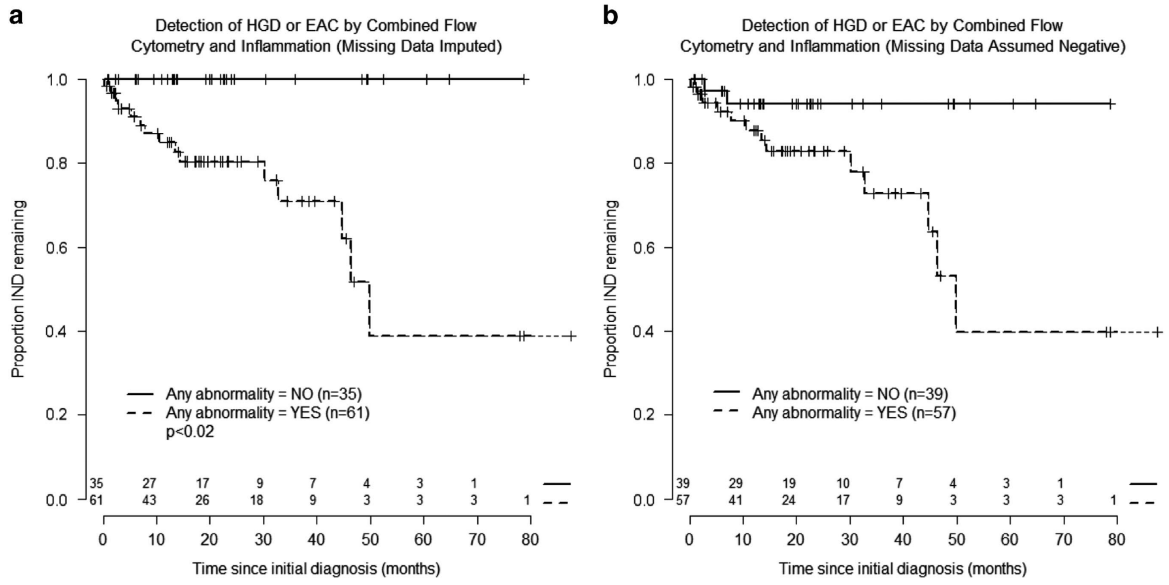

Figure 4 (**a**) Detection of HGD or EAC in IND patients with either a DNA flow cytometric abnormality or active inflammation at baseline, using a multiple imputation (unbiased) method; and (**b**) detection of HGD or EAC in IND patients with either a DNA flow cytometric abnormality or active inflammation at baseline, assuming that patients without DNA flow cytometric data had no DNA content abnormalities at baseline. This assumption increases the sample size relative to the Figure 3d (thus, providing more statistical power to reject the null hypothesis for the combined markers), but diminishes the true difference between groups due to measurement error. Each tick represents a person being censored, and the number of people remaining at risk at each 10-month interval is indicated below that axis. The estimates after 60 months have wide CIs (thus more uncertainty). CI, confidence interval; EAC, esophageal adenocarcinoma; HGD, high-grade dysplasia; IND, indefinite for dysplasia.

positive predictive value (95% CI = (76%, 96%)). In addition to this assessment of joint predictive capacity, we assessed the independent contribution of DNA flow cytometric abnormalities and active inflammation by entering both simultaneously in the Cox model (using patients with complete data only), and both were independently associated with subsequent detection of dysplasia or EAC with respective HR = 9.7 ($P = 0.002$, 95% CI = (2.3, 40.1)) and HR = 4.9 ($P = 0.02$, 95% CI = (1.4, 17.8); Table 2).

As the presence of longer BE segment, nodule/nodularity, and hiatus hernia have been previously reported as major risk factors for the development of BE, dysplasia, and/or EAC,[1,20,21] the available endoscopic reports for our cohort of IND patients were reviewed. Interestingly, the length of BE segment (HR = 1.4, $P = 0.43$, 95% CI = (0.6, 3.4)), nodule/nodularity (HR = 1, $P = 0.97$, 95% CI = (0.2, 4.2)), and hiatal hernia (HR = 0.55, $P < 0.55$, 95% CI = (0.5, 3.7)) were not associated with subsequent detection of dysplasia or EAC within the study period. Our finding that 63% of this cohort had hiatal hernia (Table 2) is consistent with previous reports that the presence of hiatus hernia is a major risk factor for the development of BE.[1,21] However, it should be noted that because the study was designed to identify factors predictive of subsequent detection of neoplasia occurring within 3 years of initial IND biopsy, we cannot completely exclude the possibility that the presence of longer BE segment, nodule/nodularity, and/or hiatal hernia may be associated with an increased risk of subsequent detection of neoplasia with a longer follow-up time.

Detection of HGD or EAC. The 1-, 2-, and 3-year detection rates of HGD or EAC were 10%, 13%, and 20%, respectively (95% CIs = (3%, 16%), (5%, 20%), and (8%, 31%), Figure 3a). The presence of active inflammation (in 46 of 96 patients) was also found to be a significant predictor of subsequent finding of HGD or EAC (Figure 3b,Table 2). The univariate HR for the detection of HGD or EAC associated with active inflammation was estimated to be 5.8 from the Cox model ($P = 0.003$, 95% CI = (1.6, 21.4); Table 2). Patients with aneuploidy had an estimated HR of 3.8 that was close to being statistically significant ($P = 0.13$, 95% CI = (0.6, 23.7)), whereas patients with elevated 4N fraction did not show an elevated risk of subsequent detection of HGD or EAC in this sample (HR = 1.2, $P = 0.84$, 95% CI = (0.2, 7.3); Figure 3c, Table 2). However, there were only five individuals in whom HGD or EAC was detected, among those who had DNA flow cytometric data available, limiting the power of statistical tests and estimation of the effects of DNA flow cytometric variables. For patients who had either DNA content abnormalities or active inflammation, the difference in the detection rate between those with subsequent neoplasia and those without neoplasia was close to being statistically significant ($P = 0.11$), but the HR was not estimable with the complete data only (infinite; Figure 3d), as none of the patients who had normal DNA flow cytometry without active inflammation at baseline were subsequently found to have HGD or EAC. The imputation-based HR estimate was 10.3 ($P < 0.02$, 95% CI = (1.7, 63); Figure 4a, Table 2). The imputation results were consistent with the expected values based on misclassification/conservative classification of patients with missing DNA flow cytometric data as negative for DNA content abnormalities (Figure 4b); the former gives somewhat better separation between groups than the latter (HR = 4.8 for the latter). The length of BE segment (HR = 2.5, $P = 0.19$,

95% CI = (0.6, 10.4)), nodule/nodularity (HR = 1.1, $P = 0.93$, 95% CI = (0.1, 8.9)), and hiatal hernia (HR = 1.4, $P < 0.66$, 95% CI = (0.3, 6.9)) were not significantly associated with an increased risk of subsequent detection of neoplasia.

DISCUSSION

EAC is theorized to progress sequentially from metaplasia to dysplasia and ultimately to EAC.[1,21] Most patients are followed up by endoscopic biopsy surveillance, and the degree of dysplasia usually determines the interval between endoscopic procedures.[2,3] The epithelial atypia should involve not only the crypts but also the surface epithelium to meet the criteria for a diagnosis of dysplasia in Barrett's metaplasia.[1] The recognition of HGD or early EAC usually prompts either resection or nonsurgical ablation therapy.[2,3,12]

Although a diagnosis of IND may very well harbor a neoplasia and portend an increased risk of subsequently developing malignancy, there are few reports regarding the natural history of IND. Data on the neoplastic risk of IND have usually been provided within the context of larger dysplasia studies, which suggest that the risk of progression from IND to HGD or EAC ranges from 2 to 12% (with a mean follow-up of 3 years).[13,14] However, the sample sizes and follow-up time were very limited in these studies, and none has assessed the potential association between active inflammation or DNA flow cytometric abnormalities and detection of subsequent dysplasia or EAC. In addition, even when the surveillance strategy follows the Seattle protocol, with four-quadrant biopsies taken in every centimeter of columnar-lined esophagus, the majority of the esophageal mucosa is not sampled. Therefore, on the basis of sampling differences alone, it is not possible to distinguish progression from detection. In other words, a patient with IND on one set of biopsies might have unsampled neoplasia elsewhere in the esophagus. For this reason, we are careful to describe the finding of neoplasia within 4 years of the biopsy finding of IND as early detection. Some of these patients may represent true progression in the form of subsequent neoplastic development; however, the finding of neoplasia within 3–4 years of the IND biopsy may represent sampling differences. Our data show that 20% of IND had detection of HGD or EAC within 3 years, and that active inflammation and DNA flow cytometric abnormalities are significant predictors of high-grade neoplasia on subsequent biopsies. Furthermore, a combination of DNA flow cytometric abnormality and active inflammation is even more highly predictive of detection of high-grade neoplasia within 3 years (Table 2).

The diagnostic category of IND is used most often in the setting in which there is cytological atypia suggestive of possible dysplasia, but with associated inflammation, ulceration, or technical issues, including lack of surface epithelium, marked cautery effect, or tangential sectioning, which limits evaluation of the surface or of the biopsy.[1] Interestingly, our data demonstrate that active inflammation, although creating diagnostic problems, is a significant risk factor for subsequent dysplasia or EAC (Figures 1b and 3b,Table 2), suggesting that active inflammation may serve as an important histologic marker for the detection of unsampled high-grade neoplasia. Although one may argue that active inflammation may be simply interfering with the reading of true dysplasia, the fact that many patients with active inflammation at baseline are found to have dysplasia several months to years afterwards further highlights the importance of active inflammation as a major risk factor for high-grade neoplasia. As such, close follow-up is warranted for patients with IND in the setting of significant active inflammation.

Aside from morphologic evaluation of dysplasia, evaluation of DNA content by flow cytometry has shown consistent results as a strong predictor of EAC. Reid *et al.*[16] reported that 9 of 13 patients who showed aneuploidy or increased G2/tetraploidy populations in their initial DNA flow cytometric analysis developed HGD or EAC on follow-up (for a mean interval of 34 months), whereas none of 49 patients without these abnormalities progressed to HGD or EAC. Our data extend these observations by showing that there is a significant correlation between the abnormal results of the DNA flow cytometric study and IND that were found to have dysplasia or EAC within 3 years. Both aneuploidy and elevated 4N fraction were associated with an increased risk in IND patients (Figure 1c,Table 2). The data further suggest that the use of flow cytometry for the analysis of nuclear DNA content and cell cycle parameters could have an important role in identifying a subset of IND patients with a higher risk of HGD or EAC, thus strengthening the vigilance for surveillance endoscopy in this high-risk group. More importantly, when active inflammation and abnormal DNA flow cytometric results were considered together, the sensitivity of the combined markers for the detection of LGD, HGD, or EAC within 3 years was 100% (95% CI = (91%, 100%)) with a specificity of 60% (95% CI = (31%, 83%)), 100% negative predictive value (95% CI = (61%, 100%)), and 89% positive predictive value (95% CI = (76%, 96%)).

The management of IND with endoscopic surveillance is variable among different institutions. Some are treated more aggressively and re-biopsied in 3–6 months, whereas others are treated similarly to those with LGD with annual endoscopies until no dysplasia is detected. As such, the clinical implications of our findings may largely depend on how this IND category is utilized for clinical follow-up and therapy. On the basis of our findings showing that patients with active inflammation and/or abnormal DNA flow cytometric results have dysplasia or EAC on biopsies taken within 3 years of the IND finding (Figures 1d and 3d), an IND diagnosis may warrant shorter follow-up surveillance intervals, especially in the setting of active inflammation and DNA content abnormalities, to ensure that no higher grade of dysplasia is present in the esophagus. Most experts use HGD as a threshold for therapeutic intervention or intensive surveillance.[3] As such, any areas of active inflammation within the BE segment should be sampled. Conversely, our data indicate that the subset of patients with IND but without active inflammation or flow cytometric DNA content abnormalities can be spared from repeat endoscopic surveillance for at least 3 years.

One limitation of our study is that only 39 (41%) of the 96 IND patients had concurrent DNA flow cytometric analysis, primarily because there is no established guideline on the appropriate use of DNA flow cytometry for the management of BE patients. However, despite the small sample size with complete DNA flow cytometric data, the statistical power

proved to be sufficient, as our results still showed a statistically significant association with subsequent detection of LGD, HGD, or EAC without imputation as well as with both endpoints (LGD/HGD/EAC and HGD/EAC) with imputation. For the combined markers that used either a DNA flow cytometric abnormality or active inflammation, only 23 patients had uncertain classification due to missing DNA flow cytometric data. Furthermore, a conservative analysis that classifies patients with missing DNA flow cytometric measurements as negative for DNA content abnormalities may be more representative of most community and academic clinical practices that may not have easy access to a DNA flow cytometry laboratory. Clinicians are still faced with management decisions in the absence of this information, and the misclassification/conservative analyses (Figures 2 and 4) demonstrate the high predictive value that can be expected, on average, when some patients are missing DNA flow cytometric data and active inflammation is considered (HR = 5.7 for detection of LGD, HGD, or EAC under misclassification; HR = 4.8 for detection of HGD or EAC under misclassification). In other words, more frequent endoscopic surveillance can be justified for the early detection of neoplasia in IND patients with active inflammation, despite missing DNA flow cytometric data. However, it should be noted that an imputation (unbiased) method allows one to avoid bias from missing data but does not allow one to makeup for the variability (large CIs) that is inherent in a smaller sample size. Yet, the median time to both endpoints (LGD/HGD/EAC and HGD/EAC) would still be centered on the correct estimate.

Another possible limitation of our study is that all the IND patients in this cohort were referred to or seen at the University of Washington and Harborview Medical Centers, which implies that referral bias cannot be ruled out, but the direction of such bias, if it exists, is difficult to predict in this situation. Despite these possible limitations, the data presented here provide important information regarding the early detection of dysplasia or EAC in a subset of IND patients with active inflammation and/or DNA flow cytometric abnormalities. Considering that the management of IND with endoscopic surveillance and/or medical treatment (including proton pump inhibitor) varies depending on the patients' symptoms, medication dose/compliance rates, and other variables, the significance of our results seems even more remarkable, because this may be much more representative of what is happening in the real world. In conclusion, our findings support the use of both histology and DNA flow cytometry to identify a subset of patients with IND who may be at increased risk for subsequent detection of dysplasia or EAC. These findings would support the further study on the utility of more frequent endoscopic surveillance for the early detection of dysplasia or EAC in patients with IND.

CONFLICT OF INTEREST

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Study Highlights

WHAT IS CURRENT KNOWLEDGE

- ✓ Dysplasia arising from Barrett's esophagus precedes esophageal adenocarcinoma (EAC).
- ✓ Cases that are difficult to diagnose as dysplastic may be designated "indefinite for dysplasia (IND)".
- ✓ There are few reports that have specifically evaluated the outcome of IND.
- ✓ Neither the American College of Gastroenterology nor the American Gastroenterological Association's practice guidelines have specific recommendations for the management of IND.

WHAT IS NEW HERE

- ✓ The 1-, 2-, and 3-year detection rates of neoplasia (including low-grade dysplasia, high-grade dysplasia (HGD), or EAC) were 25%, 37%, and 47%, respectively.
- ✓ Twenty percent of IND cases had detection of HGD or EAC within 3 years.
- ✓ Active inflammation and DNA flow cytometric abnormalities are significant predictors of neoplasia on subsequent biopsies.
- ✓ A combination of DNA flow cytometric abnormality and active inflammation is even more highly predictive of detection of neoplasia within 3 years.

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